In Vitro Evaluation of Antimicrobial Activity of *Tinospora cordifolia* & *Calotropis* species

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SYNOPSIS

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1. INTRODUCTION

The number of multi-drug resistant microbial strains and the appearance of strains with reduced susceptibility to antibiotics are continuously increasing. Therefore, there is need to search new infection-fighting strategies to control microbial infections (Sieradzki *et al*, 1999). Every culture on earth has relied on the vast variety of natural chemistries' found in plants for their therapeutic properties (Seyyed *et al*., 2010). This increase has been attributed to indiscriminate use of broad-spectrum antibiotics, immunosuppressive agent, intravenous catheters, organ transplantation and ongoing epidemics of HIV infection (Graybill, 1988; Ng, 1994; Dean and Burchard, 1996; Gonzalez *et al*, 1996). In addition, in developing countries, synthetic drugs are not only expensive and inadequate for the treatment of diseases but also often with adulterations and side effects. Despite the remarkable progress in the preparation of synthetic drugs, over 25% of prescribed medicines in industrialized countries are derived directly from plants (Newman *et al*., 2000). The World Health Organization (WHO) also considers phytotherapy in its health programs and suggests basic procedures for validation of
drugs from plant origin (Anushia *et al*., 2009) and estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicine (Tripathi and Tripathi, 2003). Medicinal plants are rich sources of antimicrobial agents. Plants are used medicinally in different countries and are the source of potential and powerful drugs (Sivastava *et al*, 1996). A wide range of medicinal parts are used to get different rasayanas which possess different medicinal properties against different microbes. Although hundred of plants species have been tested for antimicrobial properties, the majority of these have not been adequately evaluated (Balandrin *et al*, 1985).

The aim of this study was to evaluate the antimicrobial activity of two medicinal plant used in Ayurveda and traditional medicinal system for treatment of manifestations caused by microorganisms. Therefore, extracts of the following two plants from different families were tested for their potential activity against microbial pathogens: *Tinospora cordifolia* and *Calotropis* species.

Antimicrobials of plant origin

A wide range of substances used in traditional medicine is obtained from plants that can be used to treat chronic as well as infectious diseases. A vast knowledge of how to use the plants against different illnesses may be expected to have accumulated in areas where the use of plants is still of great importance (Leelavathi *et al*., 2010; Fatima *et al*., 2011). Plant synthesizes a wide variety of chemical compounds, which can be sorted by their chemical class, biosynthetic origin and functional groups into primary and secondary metabolites. Primary metabolites make up the physical integrity of the plant cell and are involved with the primary metabolite process of building and maintaining of living cells. Secondary metabolites do not seem to be vital to the immediate survival of the plant that produces them and are not an essential part of the process of building and maintaining living cells (Sharanabasappa *et al*., 2007). These secondary metabolites of plants serve as self defense mechanism against predation by many microorganisms, insects and herbivores (Vaghasiya *et al*., 2011). A growing body of evidence indicates that secondary plant metabolites play critical roles in human health and may be nutritionally important (Xiao-Ya *et al*., 2012). The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins, phenolic compounds, steroids, saponins and glycosides (Duraipandiyan *et*

al., 2006; Yadav and Agarwala, 2011). The steadily increasing microbial resistance to existing drugs is a serious problem in antimicrobial therapy and necessitates continuing research into new classes of antimicrobials (Essawi and Srour, 2000; Woodford, 2003). One way to prevent antibiotic resistance of pathogenic species is to use new compounds that are not based on existing synthetic antimicrobial agents (Shah, 2005; Chanda *et al*., 2011). Plant and plant derived agents have long history to clinical relevance as a source of potential chemotherapeutic agents (Cushnie and Lamb, 2005). Many studies have been undertaken with the aim of determining the antimicrobial and Phytochemical constituents of medicinal plants and using them for the treatment of both topical and systemic microbial infections as possible alternatives to chemical synthetic drugs to which many infectious microorganisms have become resistant (Chopra, 2007; Kumar *et al*., 2011). The antimicrobial activity of plant extracts is due to different chemical agents in the extract. These compounds are usually the secondary metabolites, which function to attract beneficial and repel harmful organisms, serve as phytoprotectants and respond to environmental changes in plants. In humans, however the compounds have beneficial effects (Johanna, 2003; Maiyo *et al*., 2010).

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2. OBJECTIVES OF PRESENT INVESTIGATION

Tinospora cordifolia and *Calotropis procera* are the plants profusely used in Ayurveda and other traditional system of medicines to cure both infectious and degenerative diseases. Lack of earlier reports on the use of leaves and latex of these plants showing antimicrobial activities in offered sufficient scope to undertake this research work.

Selection of a single suitable solvent which shows these activities is of commercial importance and is critical for the development of products of health benefits. In vitro study is the initial stage of any study and in vivo study involving experimental animals is the next stage by which one can be sure of proving the activity of the plant extracts.

This natural scientific inquisitiveness was ardently attended by undertaking the in vivo investigation of the plant extracts in animal models.

The present investigation was carried out with the leaves of these plants to investigate the antimicrobial activities with the following objectives:

- To evaluate the in vitro antimicrobial activities of the extracts of *Tinospora cordifolia*
- To evaluate the in vitro antimicrobial activities of the extracts of *Calotropis* species

The results of scientific pursuit carried out in this regard have been presented in two chapters for the convenience of reading:

1. In vitro antimicrobial activity studies of *Calotropis* species

2. In vitro antimicrobial activity studies of *Tinospora cordifolia*

3. SIGNIFICANCE OF RESEARCH WORK

In recent years, ethno-botanical and traditional uses of natural compounds, especially those of plant origin, have received much attention as they are well known for their efficacy and are generally believed to be safe for human use.

1. Antibacterial activity measured in term of diameter of zone of inhibition, the antibacterial activity of *Calotropis Species* (White & Yellow) and *T. cordifolia* on the test organisms using water, methanol and ethanol and acetone extracts of leaves of and fruit and bark exhibited antibacterial activity against both the Gram positive and Gram negative bacteria.

2. *Calotropis* species and *Tinospora cordifolia* show that it is a popular remedy in a variety of ethnic groups, as well as Ayurvedic and traditional practitioners for the treatment of a range of ailments. Researchers are exploring the therapeutic potential of this plant as it is likely to have more therapeutic properties than are currently known.

3. The inhibitory effect of *Calotropis* species was more pronounced in the latex than the leaf. It was observed that the leaf extract could be said to be bacteriostatic while the latex extract exhibited bactericidal effects. The bactericidal activity *of C. procera* latex could be due to the presence of calactin, mudarin and protein called calotropain which are active constituents of *C. procera* latex.

4. The therapeutic efficacy of *Tinospora cordifolia* extensively used in Indian System of Medicine (ISM) has been established through modern testing and evaluation (pre-clinical and clinical trials) in different disease conditions. These studies place this indigenous drug a novel candidate for bioprospection and drug development for the treatment of such diseases as cancer, liver disorders, ulcers, diabetes, heart diseases and postmenopausal syndrome, etc. where satisfactory cure managements are still not available.

5. Many infectious agents are becoming resistant to currently used antibiotics, in such cases this plant based formulation can be used to enhance the immune response of the patient and bring the cells at the site of infection and increase its killing capacity. In other disease conditions like recurrent UTI infections, this immuno stimulatory activity of the *T. Cordifolia* will be useful to flush off the adhering infectious organisms from the mucosal surfaces by enhancing the phagocytizing activity of the cells of the immune system.

6. *T. cordifolia* and *C. Procera* reveal that it is an excellent drug, which could be a good remedy for various ailments of animals as well as human beings.

4. REVIEW OF LITERATURE

Plants have long been the principal tools of traditional medicinal system. Although ancient in origin, many traditional medical paradigms and their pharmacopoeias have evolved in to quite sophisticated system, using thousands of plants and their natural system. The rural folk and tribals in India even now depend largely on the surrounding forests for their day to day needs. Medicinal plants are being looked upon not only as a source of health care but also as a source of income. India has a rich diversity of medicinal plants. Extensive study has been done by various workers for the antibacterial and antifungal activities of various plant extracts from time to time. Although there is no authentic record of medicine used by ancient people, yet Rig-Veda which is the oldest book in the library of man provides enquisitive information about the medicine used by them. Atharvaveda, another religious book of Hindus has described about 2000 plants having medicinal value. Sushruta Samhita (1000 BC) further record 700 plants of medicinal properties. Beside these there have been a number of workers from time to time who have described the medicinal importance of plants namely Charak, Watts, Kirtikar & Basu, Nandkarni, Chopra etc.
Extensive review of literature has indicated an accumulation of voluminous literature on functional properties in various spices, vegetables, fruits and herbs. The present review encompasses an up to-date published literature from various sources on different aspects of the selected three plants *viz., Tinospora cordifolia* and *Calotropis procera*. However, relevant earlier works from other plant sources have been copiously cited for critical evaluation and meaningful conclusion of our results. Our study aimed at identifying the antimicrobial activities of the selected two plants (*Calotropis* species and *T. cordifolia).* The results from the present study would aid in further studies on isolation and characterization of these compounds.

4.1 *Tinospora cordifolia*

Tinospora cordifolia commonly known as Amrita (Guduchi) is a widely used plant in folk and Ayurvedic systems of medicine. In Hindi, the plant is commonly known as *'Giloya'* which is a Hindu mythological term that refers to the heavenly elixir which has saved celestial beings from old age and kept them eternally young. *'Guduchi'*, the Sanskrit name means one which protects the entire body. The term *'Amrita'* is attributed to its ability to impart youthfulness, vitality and longevity (Bhandari, 2006).

Classification (Pullaiah, 1998)

The taxonomical hierarchy of *T. cordifolia* is as follows:

Kingdom : Plantae
Division : Angiosperma
Class : Dicotyledonae
Order : Ranunculales
Family : Menispermaceae
Genus : Tinospora
Species : cordifolia

Vernacular names of *Tinospora cordifolia*

Vernacular names of *T. cordifolia* in different parts of India are given in Table.

State	Language	Vernacular Name
Karnataka	Kannada	Amrita Balli
Tamilnadu	Tamil	Shindilakodi
Andhra Pradesh	Telugu	Tippaatigo
Kerala	Malayalam	Ambrithu
Uttar Pradesh	Hindi	Giloya, Amrita
Maharashtra	Marathi	Gulavel
West Bangal	Bengali	Gulancha
Gujarat	Guajarati	Galo

Table 1: Vernacular names of *Tinospora cordifolia*

A. Botanical description

Tinospora cordifolia is a large, deciduous, extensively spreading and climbing shrub with several elongated twining branches. Stems are fleshy with thin, grayish or creamy white colored bark and with long, filiform and fleshy aerial roots from the branches. Leaves are simple, alternate, ovate or ovate chordate which is 10-20 cm long and 8-15 cm broad. The flowers are unisexual, small on separate plants and greenish yellow. In axillary and terminal racemes or racemose panicles, the male flowers are clustered and female flowers are usually solitary. The drupes are ovoid, glossy, succulent, red, pea sized and occur in winter (Khosa and Prasad, 1971; Chopra, 1994). The seeds are curved. Fruits are fleshy and single seeded. Flowers grow during the summer and fruits during the winter (Kirtikar and Basu, 1975).

B. Origin and distribution

Tinospora cordifolia is indigenous to the tropical areas of India, Myanmar and Sri Lanka ascending to an altitude of 1200 m. It is a fairly common plant of deciduous and dry forests, growing over hedges and small trees (Kirti *et al.*, 2004; Bhandari, 2006).

C. Biophysical limits

They grow at low altitude up to 1030 m and preferably grow at sandy loam.

D. Uses of *Tinospora cordifolia* in traditional system of medicine

Tinospora extracts are widely used in the traditional system of medicine in the treatment of jaundice, rheumatism, intermittent fevers, eye and liver ailmentanti-spasmodic, anti inflammatory, diabetes, seminal weakness, urinary tract infections, fever, general debility, skin diseases, and expectorant, carminative, digestive, antistress and aphrodisiac.. It is also an important constituent of many Ayurvedic formulations and is reported to possess adaptogenic and immunomodulatory activity in fighting infections (Nayampalli *et al.,* 1982). The stem is bitter stomachic, diuretic (Nayampalli SS et al., 1988), stimulates bile secretion, causes constipation, allays (satisfies) thirst, burning sensation, vomiting, enriches the blood and cures jaundice. (Mishra p et al, 2014). The root and stem of *T.cordifolia* are prescribed in combination with other drugs as an antidote in snake bite and scorpion sting (Nadkarni KM et al., 1976; Zhao TF et al., 1991). Oral administration of an aqueous T. Cordifolia root extract to alloxan diabetic rats caused a significant reduction in blood glucose and brain lipids (Dhaliwal KS et al., 1999).). Extract of *T. cordifolia* has also exhibited in vitro inactivating property against Hepatitis B and E

surface antigen (Mehrotra R et al., 2000). In the Antibacterial activity of aqueous, ethanol and chloroform extracts of leaves and stem of *Tinospora cordifolia* Hook.F.Thoms were tested on clinical isolates of urinary pathogens *viz., Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris* and *Pseudomonas aeruginosa* by disc method. Ethanol extract of leaf showed greater inhibitory action than other tested extracts. Indian system it is known to increase the longevity and the body's resistance against various diseases (Bhatt and Bhat, 1996).

E. Current status

A number of chemical constituents belonging to different groups, *viz.*, terpenoids like tinosporide (Khuda *et al*., 1964), tinosporaside (Hanuman *et al*.,1986), furanolactone diterpene (Khan *et al*., 1989), alkaloids like tinosporine (Khuda *et al*., 1964), berbrine (Pachey and Schneidir, 1981) and steroids like β sitosterol (Khaleque *et al*., 1970) have been isolated from *T. cordifolia* stem and roots. Some of the functional properties attributed to *T. cordifolia* are as follows:

Anticancer activity

Administration of intraperitoneal injection *T. cordifolia* stem methanol extract to BALB/c mice (200 mg/kg b.w.) for five days increased the total WBC count significantly. It reduced solid tumor growth and synergistically acted with cyclophosphamide in reducing the animal tumors (Mathew and Kuttan, 1999).

Antidiabetic and antihyperglycemic activity

Alcoholic extracts of the stem at a concentration of 50, 100 and 200 mg/kg b.w. caused a reduction in the fasting blood sugar in the alloxan induced diabetic rabbits (Wadood *et al*., 1992). Daily oral feeding of *T. cordifolia* extracts for 40 days in streptozotocin diabetic mice caused a reduction in blood glucose concentration and also prevented polyurea (Grover *et al*., 2002).

Antiinflammatory activity

The stem extract of *T. cordifolia* showed anti-inflammatory activity on carrageenin induced hind paw edema in rats (Sharma and Singh, 1980).

Antioxidant activity

Alcoholic extract of *T. cordifolia* roots administered at a dose of 100 mg/kg b.w. orally to diabetic rats for six weeks normalized the antioxidant status of liver and kidney (Prince *et al*., 2004).

Hypolipidaemic activity

Administration of aqueous extract of roots of *T. cordifolia* at a concentration of 5 g/kg b.w. for six weeks resulted in a significant reduction in serum and tissue cholesterol phospholipids and free fatty acids in alloxan diabetic rats (Stanely *et al*., 1999).

Immunomodulatory activity

The water and ethanol stem extracts of *T. cordifolia* inhibited immunosuppression */produced by cyclophosphamide (Manjreker *et al*., 2000).

Hepatoprotective activity

The hepatoprotective activity of *T. cordifolia* extract has been demonstrated in CCl4 induced liver damage in rats. The extract was found to normalize the liver function as assessed by morphological, biochemical and functional tests (Bishayi *et al*., 2002).

Tinospora cordifolia was selected for our study due to its various functional properties as listed above and also due to its wide usage in Ayurvedic formulations. *In vivo* and *in vitro* research investigations on *T. cordifolia* have shown the use of its stem and roots and not the leaves. Not

many studies have been carried out to show the antimicrobial, antioxidant and antidiabetic activities of its leaves. Antifungal activity studies against dermatophytes have not been proved till now even though its activity against the field fungi have been carried out. Moreover, in this study we are trying to select a suitable solvent extract of leaves which has antimicrobial, antioxidant and antidiabetic activity. All these facts have prompted us to select only the leaves of *T. cordifolia* for our study.

Table.2: Phytochemical Profile of *T.cordifolia*

Chemical Class	Phytoconstituents	Plant Part
Alklloids (Singh SS et al.,2003; Sinha K et al., 2004)	Berberine, Tembeterine	Stem
	Choline,Tinosporin,isocolumbin,tetrahydro palmitine,jatrorrhizine	Root
	Palmitine	Stem and root
Glycosides (Singh SS et al., 2003; Gagan VD et al., 1994; Wazir V et al., 1995 ; Gagan VD et al., 1996; Maurya R et al.,1997; Ghosal S et al.,1997)	18-norclerodane glucoside, Furanoid dilterpene glucoside, cordiofolioside A, cordiofolioside B, palmatosides C, palmatosides P1, cordiofolioside C, cordiofolioside D, cordiofolioside E	Stem
Diterpenoid Lactones (Singh SS et al., 2003; Maurya R et al., 1997; Maurya R et al., 1989; Swaminathan K et al., 1989)	Clerodane derivatives, tinosporon, tinosporides, jaiterine, columbin	Whole Plant
Sesquiterpenoids (Maurya R et al.,1998)	Tinocordifolin	Stem
Steroids (Singh SS et al., 2003)	sitosterol,-sitosterol,20 -hydroxyecdysone, ecdysterone,makisterone A, giloinsterone	Aerial Parts Stem
Aliphatic compounds (Singh SS et al., 2003; Thippeswamy G et al., 2008)	Octacosanal, heptacosanol, nonacosan-15-one	Whole Plant
Miscellaneous Compounds (Singh SS et al.,2003; Hamuman JB et al., 1986)	Tinosporidine, Cordifol,cordifelone, N-trans-feruloyltyramine as diacetate, giloin, gilonin, tinosporic acid	Root

***Calotropis* species**

In India, the genus is represented by two species. viz. *Calotropis gigantea* and *Calotropis procera*. The first species being abundant while the second being restricted to forest areas. These plants are commonly known in English as Giant Milk Weeds or Swallow-worts. This species is one of the special classes of plants that can avoid or repel the grazing animals (Sastrt et al, 1990).

Classification:
The taxonomical hierarchy of *Calotropis procera* and *Calotropis gigantea* is as follows:

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Magnoliopsida
Family	Asclepiadaceae
Genus	*Calotropis*
Species	*procera*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Magnoliopsida
Family	Asclepiadaceae
Genus	*Calotropis*
Species	*gigantea*

A. Botanical description:
Calotropis procera is a shrub or small tree up to 2.5 m (max. 6m) high and *Calotropis gigantea* is a shrub about 6 m in height. Stem usually simple, rarely branched, woody at the base and covered with a fissured, corky, branches somewhat succulent and densely white tomentose, early glabrescent. Leaves are opposite, simple, sub sessile, stipule absent; blade oblong-obviate to broadly obviate, 5-30x 2.5-15.5 cm, apex abruptly and shortly acuminate to apiculate, base cordate, margins entire, succulent. They are white tomentose when young, later glabrescent and glaucous. Inflorescence is Dense, multiflowerd, umbellate cyme arising from the nodes and appearing axillary or terminal. Flowers are Hermaphrodite, pentamerous, pedicle 1-3 long; calyx 5-lobed, shortly united at the base, lobes ovate, 4-7x 3-4 mm, glabrescent. Fruits are Simple, fleshy, inflated, and subglobose to obliquely ovoid follicle up to 10 cm or more in diameter. Seeds are numerous, flat obviate, 6x 5 mm, with silky white pappus 3 cm or more long. These plants are reproduced via cross pollination through insect such as monarch butterflies. However, both animals and wind also dispers seeds. Progeny are genetically divergent from its parents (chromosome number 2n = 22).

B. Geographical Distribution
Calotropis procera Native: Afghanistan, Algeria, Burkina Faso, Cameroon, Chad, Cote d' Ivoire, Democratic Republic Of Congo, Egypt, Eritea, Ethiopia, Gambia, Gana, Guinea-Bissau, India, Iran, Iraq, Israel, Kenya, Kuwait, Lebanon, Libyan Arab Jmahiria, Mali, Mauritania, Morocco, Mozambique, Myanmar, Nepal, Nigeria, Oman, Pakistan, Saudi Arabia, Senegal, Sierra Leone, Somalia, Sudan, Syrian Arab Republic, Tanzania, Thailand, Uganda, United Arab Emirates, Vietnam, Yemen and Republic Of Zimbabwe.
Calotropis gigantea is a wasteland weed better known as milkweed, habitat of Asian countries that includes, India, Indonesia, Malaysia, Philippines, Thailand, Sri Lanka and
China.

C. **Biophysical Limits**
These plants grow at altitude up to 1300 m where a mean annual rainfall ranges, 300-400mm. They are preferably distributed in sandy soils.

D. Uses of *Calotropis procera* in traditional system of medicine
Compounds derived from the plant have been found to have emeto-cathartic and digitalic properties. The principal active medicinal compounds are asclepin and mudarin. Other compounds have been found to have bactericidal and vermicidal properties. It is used to treat boils, infected wounds and other skin problems in people and to treat parasitic skin infestation in animals (Himalaya, 2002). It also yield ash for making gun powder, the latex is processed and

use in treating vertigo, baldness, hair fall, tooth aches, intermittent fevers, rheumatoid/joints swellings and paralysis (Vohra, 2004). The whole plant when dried and consumed is a good tonic, antihelmintic and as an expectorant (Agharkar, 1991; Warrier et al., 1996). The roots, besides being endowed with similar qualities serves as an effective laxative. Traditionally, the dried root is powdered and effectively used to cure bronchitis, asthma, leprosy, eczema and elephantiasis, hepatic and splenic enlargement (Vohra, 2004). The milky juice is regarded as drastic purgative and caustic flowers were considered to improve digestion, catarrh and increases appetite (Oudhia, 2001). The pungent latex extracted from the leaf and flowers of C. procera is processed and used in the commercial preparation of eye tonic (Vohra, 2004). The juice was also found to induce abortion in women and tanners use the milky juice to remove hair from the hides (Singh *et al*., 1996). Traditionally, the leaves of the plant are warmed and tied around any body organ in pain. It practically useful in backache and joint pains, warm leaves also relieve from stomach ache if tied around. Inhalation of burnt leaf cures headache. The traditional folk healers use the milky latex of C. procera for several ailments. Leaf latex if applied on fresh cut, stops bleeding immediately. Recent investigation has found that the alkaloids calotropin, calotaxein and uskerin are stimulant to heart (Ashwari, 2009). It is also used by traditional medicine practitioner in Gwari communities for the treatment of ring worms (Kuta, 2008). Leaves, roots, stem, flowers and latex of C. gigantea are used in traditional medicinal system to cure several diseases and medicinal potential of the C. gigantea proved scientifically.

E. Current status

A number of chemical constituents belonging to different groups have been isolated from *C. procera* stem and roots and latex. Some of the functional properties attributed to *C. procera* are as follows:

Pharmacological activities

The plant has attracted much attention due to following biological activities: The previous pharmacological studies include reports of anticancer, antifungal (Hassan *et al*, 2006) and insecticidal activity of C. procera. The flowers of the plant exhibit hepatoprotective activity (Setty *et al*, 2007), anti inflammatory, antipyretic, analgesic, and antimicrobial effects and larvicidal activity (Markouk *et al*, 2000; Mascolo *et al*, 1989). The latex of the plant is reported to possess analgesic and wound healing activity (Dewan *et al*,2000 ; Rasik *et al*,1999), as well as anti-inflammatory (Arya,2005) and antimicrobial activity (Sehgal *et al*,2005) while the roots are reported to have anti-fertility (Kamath *et al*,2002) and anti-ulcer effects (Basu *et al*, 1997).

Analgesic activity

A single oral dose of dry latex ranging from 165 to 830 mg/kg produces a significant dose-dependent analgesic effect against acetic acid-induced writhing. The effect of dry latex at a dose of 415 mg/kg is more pronounced than a 100 mg/kg oral dose of aspirin. In addition, dry latex (830 mg/kg) produces marginal analgesia in a tail-flick model which is similar to that of aspirin. It was concluded that the protein fraction derived from the whole latex of Calotropis procera possesses antinociceptive activity, which is independent of the opioid system. (Vasconcelos, 2005)

Antifertility activity

The effect of an ethanolic extract of the roots of Calotropis procera has been studied in albino rats to explore its antifertility and hormonal activities. Strong anti-implantation (inhibition 100 %) and uterotropic activity was observed at a dose of 250 mg⁄kg (1⁄4 of LD50). No antiestrogenic activity was detected (Ranab *et al*, 2002).

Anti-tumor studies

The anti-tumor potential of the root extracts of Calotropis procera Linn., was investigated using the methanolic (CM), hexane (CH), aqueous (CW) and ethyl acetate extract (CE) and its possible mechanism against Hep2 cancer cells was studied. The results of flow cytometric analysis clearly demonstrated that the root extracts produced apoptosis of Hep2 cells through cell cycle arrest at the S phase, thus preventing cells from entering the G2/M phase. The results of this study indicate that the root extracts of C. procera inhibit the proliferation of Hep2 cells via mechanisms based on apoptosis and cell cycle disruption (Mathura, 2009).

Antihelmintic activity

The antihelmintic activity of *Calotropis procera* Flowers, in comparison with levamisole, was evaluated in a series of in vitro and in vivo studies. The *in vitro* studies demonstrated the antihelmintic effects ($P<0.05$) of crude aqueous (CAE) and crude methanolic extracts (CME) of *Calotropis procera* flowers on live *Haemonchus* (*H*.) *contortus* as shown by mortality or temporary paralysis. It was found that *Calotropis procera* flowers possess good antihelmintic activity against nematodes, although this was less than that exhibited by levamisole (97.8 %–100 %) (Iqbal, 2005).

Anti-hyperglycemic effect

The dry latex (DL) of *Calotropis procera* possessing potent anti-inflammatory activity was evaluated for its antioxidant and anti hyperglycemic effects in rats with alloxan-induced diabetes. Daily oral administration of dry latex at 100 and 400 mg/kg produced a dose-dependent decrease in blood glucose and an increase in hepatic glycogen. Dry latex also prevented the body weight loss in diabetic rats and reduced the daily water consumption to values comparable with those of normal rats.(Kumar, 2005).

Inflammatory activity

Latex of *Calotropis procera* was studied for its inflammatory reactions using pedal oedema and air pouch models of inflammation in rats. Subcutaneous injection of aqueous solution (0.1 ml of 1%) of dry latex (DL) into the plantar surface of paw produced significant inflammation. (Al-Yahya, 1985).

Antimalarial activity

The ethanolic extracts of the different parts of *Calotropis procera* showed IC50 values ranging from 0.11 to 0.47 mg/ml against *P. falciparum* MRC20_CQ-sensitive. and from 0.52 to 1.22 mg/ml against MRC76_CQ-resistant strains, flowerand bud extracts being the most active. (Sharma, 2000).

Chemicals isolated from various parts of Calotropis Procera

Table.4 Chemicals isolated from various parts of *Calotropis Procera*

Plant Species/ Parts	Chemical Constituents	Year /References
Leaves and Stalk	Calotropin	1936, Hesse & Richander
Latex	Uscharin, Calotoxin, Calactin	1938, Hesse et al.
Whole Plant	Uscharine, amyrin esters, uscharidin, Calotoxin and Calactin	1950, Hesse et al.
Seed	Coroglaucigenin, Frugoside, Corotoxigenin, Calotropin	1955, Rajagopalan et al.
Latex	Cardenolite- Voruscharine	1957, Hesse & Lettenbauer
Latex	Calotropagenin	1959, Hassall & Reyle
Latex	Voruscharine	1960, Hesse and Ludwig
Latex	Proteolytic enzyme Calotropain	1961, Shukla & Krishnamurti
Latex	O- pyrocatechuic acid	1963, Ibrahim
Root Bark	α- amyrin, β- amyrin, Ψ- taraxasterol, β- sitosterol, taraxasteryl acetate, taraxasteryl benzoate, α- amyrin benzoate, β- amyrin acetate, acetic acid and isovaleric acid	1968, Anjaneyulu and Ramachandra
Leaf	Taraxasterol isovalerate	1968, Anjaneyulu and Ramachandra
Root Bark	Benzoyllineolane, Benzoylisolineolane,	1968, Chandler et al.
Latex	Uzarigenin, Syriogenin and Proceroside	1969, Brueschweiler et al.
Leaves	D- glucose, D- arabinose, D- glucosamine and α- rhamnose	1969, Qudrat-i- Khuda and Amir
Leaves	α- amyrin, β- amyrin, β- sitosterol	1969, Saber et al.
Latex	Calotropin, Calotoxin, Uscharin,Uscharidin and Choline	1971, Mahran et al.
Leaves	Asclepin	1972, Singh and Rastogi
Leaves	Calotropin and Calotropagenin	1979, Malik et al.
Leaves, Latex	Phenolic contents	1986, Marimuthu and Kothari
Flowers	Calotropenyl actate and Procestrol	1988, Khan et al.
Latex	Lupecol, β- amyrin, α- and β- calotropeol and 3- epimoretenol	1989,Khan and Malik
Flowers	Multiflorenol, Cyclosadol, Cycloart- 23- ene- 3 β, 25-diol β- sitostenone, α- and β-amyrin, Stigmasterol and β- sitosterol	1990, Khan and and Malik
Latex	Calotropin, Calactin, Calotoxin, Uscharin, Uscharidin , Voruscharin, Calotropin D I and Calotropin D II, Calotropin F I, Calotropin F II Taraxast-20 (30)-en-3 (4-methyl 3-pentenoate)	1993, Rastogi and Mehrotra
Root and Root Bark	α- and β-amyrin, taraxasterol, 4J-isomer, taraxasteryl isovalerate, taraxasteryl acetate,	1993, Rastogi and Mehrotra 2001, Ansari and Ali

Leaves and Stalk	β- sitosterol and quercetin-3- rutiniside	1929, Duke, 2002, Gallegos et al.
Latex	Calotropin, Calotropagenin, Cardenolides, stigmosterol, β- sitosterol, quercetin-3-rutinoside and triterpenoids	2003, Kumar and Jagannadham
Latex	Uzarigenin, Syriogenin, proceroside, Quercetin-3-rutinoside, lupeol, β-amyrin, α- and β- calotropeols, 3- epimoretenol and procerain	2003, Shivkar and Kumar

Table.5 Chemicals isolated from various parts of Calotropis gigantean

Sl.No.	Class of Compounds	Plant Part			Test Performed
		Flower	Bud	root	
1	Alkaloids	+	+	+	Dragendorff's test, Mayers test
2	Carbohydrates	+	+	+	Molish test, Fehling test
3	Glycosides	+	+	+	Keller killiani test
4	Phenolic compounds/tannins	+	+	+	Ferric chloride test
5	Proteins and amino acids	+	+	+	Xantho protein test
6	Flavanoids	+	+	+	Ammonia test
7	Saponins	+	+	+	With water With Na2CO3
8	Sterols	+	+	+	Liebermann-Burchard test, Salkowski reaction, Hesse'sreaction
9	Acid compounds	+	+	+	With Na2CO3, With litmus paper
10	Resins	+	+	+	With double distilled water,With acetone and conc. HCl
11	Peroxides	-	-	-	Potassium Iodide test
12	Polyuronoids	-	-	-	Haemotoxylin test

5. MATERIALS AND METHODOLOGY

4.1 Collection of plants

The young leaves and stem of *Tinospora cordifolia* and *Calotropis procera* were collected from 3-5 months old healthy plant grown in Mansa, Punjab, India, which receives a mean annual rainfall ranging from 320 to 378 mm with an average temperature of 32 C. The pH of the garden soil was 7. The plant leaves and stem were washed thoroughly with tap water followed by sterile distilled water and shade dried at room temperature for **10-15** days.

4.2 Preparation of extracts

4.2.1 Grinding of selected plant materials

The plant material was dried at 37oC for 72 hours. Exposure to sunlight was avoided to prevent the loss of active constituents. After drying the plant material was cut into pieces. The powdered plant material was taken for extraction procedure. This step was completed within **3-5** days.

4.2.2 Preparation of ethanolic extract

Stems of the plant were washed thoroughly with distilled water and shade dried. Ethanolic extract of the dried stems of *T. cordifolia* was prepared by maceration method using 50 ml ethanol. The extraction was done at room temperature for **7** days.

4.2.3 Storage conditions

The extract was stored in a cool condition protected from direct sunlight.

4.2.4 Sterility checking

Prior to subjecting the extracts to antibacterial assay they were checked for sterility by inoculating on nutrient agar and incubating at 37°C.

4.3 Selection of antibiotics

Broad spectrum antibiotics, *Penicillin* and *Tetracyclin* were used as control drugs.

4.4 Selection of bacterial strains

In this present study the test microorganisms used (bacteria: *Escherichia coli* (MTCC No.40), *Staphylococcus aureus* (MTCC No.87), *Proteus vulgaris* (MTCC No.742), *Pseudomonas aeruginosa* (MTCC No.424), *Bacillus subtilis* (MTCC No.441), were procured from MTCC Chandigarh. This procedure took time period of **20-25** days.

DETERMINATION OF ANTIMICROBIAL ACTIVITY

Antimicrobial activity was performed by standard method, disc diffusion on agar and the MIC was calculated using dilution method.

4.A Disc diffusion Method

4.A.1 Reagents for the disc diffusion test

Mueller-Hinton Agar Medium

• Mueller-Hinton Agar is considered to be the best for routine susceptibility testing of non-fastidious bacteria for the following reasons.
• It shows acceptable batch-to-batch reproducibility for susceptibility testing.
• Medium is transparent, so that the inhibition zone can be visualized clearly.
• It gives satisfactory growth of most non fastidious pathogens.
• A large body of data and experience has been collected concerning susceptibility tests performed with this medium.

Composition of Mueller-Hinton Agar

Beef, infusion form: 300gm/ liter
Casein acid hydrolysate: 17.50gm/ lit
Starch: 1.50gm/ lit
Agar: 17gm/ lit
pH: 7.3 ± 0.2 (at 25°C)

Preparation of Mueller-Hinton agar

1. Mueller-Hinton Agar was prepared from a commercially available dehydrated medium (Himedia) according to the manufacturer's instructions.
2. Immediately after autoclaving, it was allowed to cool in a water bath at 45-50℃. Before using the medium in laminar air flow the outer surface of the conical flask should wiped with cotton using 70% IPA, to avoid cross contamination.
3. Aseptically transfer about 25 to 30 ml of sterile prepared medium into 100mm dia petridishes. The pertidish should be flat-bottomed and it should be placed on a level, horizontal surface to give a uniform depth of approximately 4 mm. The agar medium was allowed to cool at room temperature, unless the plate is used in the same day, the prepared should be stored in a refrigerator at 2-8℃.
4. Plates were used within seven days after preparation unless adequate precautions, such as wrapping in plastic or paraflim, have been taken to minimize drying of the agar.
5. A representative sample of each batch of plates was examined for sterility by incubating at 30 to 35℃ for 24 hrs or longer.

Preparation of antibiotic stock solutions

Powders of two antibiotics Penicillin and Tetracycline (purity 100%) were accurately weighed and dissolved in sterile distilled water to give appropriate dilutions of about 0.5, 1.0, 1.5, 2.0 and 2.5 mg/ml to yield the required concentrations. The stocks were aliquot in 5 ml volumes and frozen at -20℃.

4.A.2 Procedure for performing the Disc Diffusion Test

The disk diffusion method allows for the simultaneous testing of a large number of antimicrobials in a relatively easy and flexible manner. In this method, the bacterial inoculum is adjusted to certain concentration, inoculated onto the entire surface of a Mueller-Hinton agar (MHA) plate with a sterile cotton-tipped swab to form an even lawn. The paper disks (6 mm in diameter; BD Diagnostic Systems) impregnated with diluted antibiotic solution was placed on the surface of each MHA plate using a sterile pair of forceps. Then the plates were incubated aerobically and the diameter of zone inhibition was measured by a ruler or caliper. Based on the diameter of the inhibiton zone and the CLSI interpretative criteria, the results are then assigned to three categories, susceptible, intermediate, or resistant. The bigger the diameter of the inhibition zone, the more susceptible is the microorganism to the antimicrobial. The major disadvantages of this method are unable to generate the MIC value (i.e., not quantitative) and difficult to examine the susceptibility of fastidious and slow-growing bacteria (Wilkins & Thiel, 1973; Dickert *et al.*, 1981). Moreover, different from antimicrobial agents used in clinical settings, there are currently no standard CLSI interpretive criteria of disk diffusion results to support natural antimicrobials susceptibility testing; thus, it is unable to explain the zone diameter generated by disk diffusion for natural antimicrobials.

Minimum Inhibitory Concentration

Different concentrations of the leaves and latex extract of *C.procera* and *T.cordifoli*a were prepared to obtain. Three drops of overnight broth culture of the test organisms were inoculated into the dilutions and incubated at 37oC for 24 hours. The lowest concentration of the extracts that inhibited the growth of the test organisms was recorded as the minimuminhibitory concentration (MIC).

6. CHAPTERS PROPOSED FOR RESEARCH WORK

Chapter 1- Introduction

Chapter 2- Review of Literature

Chapter 3- Materials and Methodology

Chapter 4- Results and Discussion

Chapter 5- Summary

Chapter 6- Bibliography

7. EXPECTED DURATION OF RESEARCH WORK

Time Period	Work
Three months	collect samples, strains and material
Three months	literature regarding research work and grow plants of interest
One year	perform research work and determination of antimicrobial activity
Six months	write Thesis work, draw tables, diagrams, calculate statistical data and binding of thesis

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Signature of Supervisor with Date **Signature of Candidate with Date**